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Cryptococcus: from environmental saprophyte to global pathogen

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Abstract

Cryptococcosis is a globally distributed invasive fungal infection caused by species within the genus *Cryptococcus* that presents substantial therapeutic challenges. Although natural human-to-human transmission has never been observed, recent work has unveiled multiple virulence mechanisms that allow cryptococci to infect, disseminate within and ultimately kill their human host. In this Review, we describe these recent discoveries that illustrate the intricacy of host-pathogen interactions and reveal new details about host immune responses that either help protect against disease or increase host susceptibility. In addition, we discuss how this improved understanding of both the host and the pathogen informs potential new avenues for therapeutic development.

Cryptococcosis has been recognized since 1894, when the pathologist Otto Busse and physician Abraham Buschke jointly identified *Cryptococcus* as the cause of a chronic granuloma of the tibial bone in a 31-year-old woman. However, human cryptococcosis only became recognized as a major health threat with the onset of the AIDS pandemic in the 1980s, in which these fungal infections became a common AIDS-defining illness in patients with catastrophically reduced T-cell function (**Box 1**). Although cryptococcosis is predominantly a disease of immunocompromised patients, a recent outbreak of cryptococcosis in otherwise healthy individuals in North America and Canada (now known as the Pacific Northwest Outbreak) has focused attention on the capacity of some lineages of the pathogen to act as primary pathogens (see below).

Since its identification, cryptococcosis has been attributed to a single fungal species, *Cryptococcus neoformans*. However, improved molecular methods led to a previous variety, *Cryptococcus neoformans* var. *gattii*, being classified as a novel species, *Cryptococcus gattii*, in 2002[1]. More recently, whole-genome sequencing-based analyses have highlighted the complex evolutionary history of this group (**Box 2**) and led to a proposal to further split *C. neoformans* into two species (*C. neoformans* and *Cryptococcus deneoformans*) and *C. gattii* into a total of five species (*C. gattii, Cryptococcus bacillisporus, Cryptococcus deuterogattii, Cryptococcus tetragattii* and *Cryptococcus decagattii*)[2]. However, as detailed biological comparisons between these five species have not been yet undertaken,

we have adopted the simpler distinction into the two species *C. gattii* and *C. neoformans* throughout this article.

Cryptococcus transmission and disease onset

In the environment, cryptococci reside in diverse ecological niches (**Box 3**). Both *C. neoformans* and *C. gattii* are abundant in decaying material within hollows of various tree species, although *C. gattii* has been suggested to favour trees with waxier cuticles (such as *Pseudotsuga menziesii*) [3, 4]. Furthermore, *C. neoformans* is globally distributed, whereas *C. gattii* has classically been viewed as a tropical or subtropical fungus. However, increased surveillance has now identified environmental reservoirs for *C. gattii* in the Northern USA, Canada and Northern Europe, indicating that this species may also have a wider ecological range than previously recognized.

C. neoformans is particularly abundant in avian excreta[4,5] and its association with feral pigeons could be a major source of infection in densely populated urban areas. In addition, both *C. neoformans* and *C. gattii* are able to survive and replicate within free-living amoebae and soil nematodes and it is possible that these alternative hosts may have an important role in determining the distribution and virulence of different cryptococcal lineages around the world (**Box 3**).

With the exception of very rare iatrogenic[6] or zoonotic[7] transmission events, naturally acquired cases of cryptococcosis are believed to start with inhalation of fungal cells from the environment. Within the lung, *Cryptococcus* species can cause pneumonia in immunosuppressed patients, but in immunocompetent hosts the fungal cells are either cleared by the immune system or establish an asymptomatic latent infection. Upon subsequent immunosuppression, this latent infection can then disseminate to other tissues, most notably the central nervous system (CNS). Once established within the CNS, cryptococcosis causes an overwhelming infection of the meninges and brain tissue that is frequently accompanied by raised intracranial pressure; without rapid and effective treatment, CNS infection is invariably fatal. Despite intensive investigations, it remains unclear whether reactivation and dissemination of long-term latent pulmonary infection is a more important cause of

cryptococcosis in patients than *de novo* acquisition from the environment, but experiments in animal models indicate that both routes are capable of causing lethal disease.

Exposure to *C. neoformans* is common in humans, as most individuals produce antibodies against this fungal species by school age[8]. During active growth, cryptococcal cells are too large to penetrate deep into the human lung and thus the initial inoculum is believed to comprise either desiccated cells or spores. The relative contribution of these two cell types to the burden of disease remains unclear, largely due to technical challenges associated with generating and purifying spores. However, recent studies have demonstrated that lethal brain infections can develop from spore inocula, that spores are readily phagocytosed by host immune cells and, interestingly, that rising humidity dramatically increases spore viability[9,10,11]. Thus, as with other fungal pathogens such as *Coccidiodes immitis,* environmental conditions may be an important factor in regulating human cryptococcal exposure.

Cryptococcal pathogenesis

Traditional virulence factors produced by *Cryptococcus* (such as the capsule and melanin production) and changes in fungal growth due to the host temperature (37°C) have been previously reviewed in great detail (see for example references [12,13]). Therefore, in this section of the Review, we will focus on recently emerging concepts in cryptococcal pathogenesis.

Fungal morphology. Whether derived from spores or yeast cells, upon inhalation into a mammalian host, all cryptococci transition to or maintain a yeast form. When grown under laboratory conditions, *Cryptococcus* cells are round and 5-7 μm in diameter. However, their cell size, structure, and characteristics can vary dramatically within the host.

The best-characterized atypical morphology of *Cryptococcus* cells is the titan cell[14] (**Figure 1**). Titan cells are greater than 12 μm in diameter (excluding the capsule), polyploid, have highly cross-linked capsules and a thickened cell wall[15,16]. Recent studies have shown that titan cells contain elevated levels of chitin. This polysaccharide is recognized and cleaved by host chitinases, which

induces a detrimental adaptive immune response (see below) [17]. Intriguingly, the polyploidy observed in titan cells enhances genetic adaptation to the stressful host environment, resulting in increased within-host survival [18].

In addition to the large titan cells, unusually small cryptococcal cells have also been observed[19,20] (**Figure 1**). These so-called "drop" or "micro" cells are only 2-4 μm in size, despite having a thickened cell wall, and appear adapted for growth within macrophages. At present, little is known about this cell type, although they appear to be relatively metabolically inactive and therefore may have an important role during the latent stage of disease.

In the environment or under laboratory conditions, cryptococci can also grow as hyphae (during sexual reproduction) or pseudohyphae, but (unlike other pathogenic fungi) these morphologies are not seen in human infections [21]. Recent studies overexpressing the transcription factor Znf2, a "master regulator" that triggers the transition from yeast to hyphal growth, showed that the hyphal form elicits a robust protective immune response and is readily cleared by the host[22,23], perhaps explaining why filamentous morphologies are not seen in mammalian infections. Interestingly, however, hyphal cryptococci are protected from predation by free-living amoebae[24] and thus mammalian and amoebal hosts presumably exert opposing selective pressures on this aspect of cryptococcal morphology (with mammalian hosts favouring the existence of the yeast forms and amoebae favouring hyphal forms).

Fungal ageing. Even within a clonal infection, not all cryptococcal cells are equal. For example, the age of individual cryptococcal cells has emerged as a factor that impacts survival in the host and subsequent pathogenesis[25]. Older cells present in the initial infection, referred to as founder cells, are better able to resist phagocytosis and killing by phagocytes and are resistant to antifungal drugs. This increased resistance to phagocyte killing and antifungals is potentially due to changes in cell wall structure [26], and results in the accumulation of founder cells in the brain at a higher frequency than young cells[27].

Population-wide signals. In bacterial infections, quorum sensing is a well-known mechanism that regulates virulence according to population density. Interestingly, emerging data suggest that quorum sensing may also have an important role during cryptococcal pathogenesis. For example, a quorum sensing effect, mediated by an oligopeptide with 11 amino acids, was identified using mutations in the global repressor TUP1. Notably, although TUP1 is present in several species, the quorum sensing effect mediated by this oligopeptide appears only to occur in *C. neoformans*[28]. However, more recently a different signaling molecule, pantothenic acid, has been demonstrated to mediate quorum sensing both between different cryptococcal strains and between cryptococci and other, relatively distantly related, fungal species[29]. The adhesin Cfl1 has also been shown to modulate colony morphology in a paracrine manner[30]. Activation of the hyphal regulator Znf2 (discussed above) induces expression of this adhesin, some of which is shed into the environment and triggers neighboring cells to activate Znf2, leading to a positive feedback loop. Thus cryptococci may communicate locally using a range of chemical messengers[31].

Perhaps most unique is the observation that light-sensing pathways may also be important for virulence in *Cryptococcus* since deletion of either *Bwc1* or *Bwc2*, which encode two transcription factors that control fungal responses to light, reduces virulence in a murine model of infection[32]. In the dark, BWC1 and BWC2 bind to DNA and repress genes involved in filamentation. However, upon light activation, they release this inhibition leading to filamentation and upregulation of UV-resistance pathways. Thus, it is possible that an additional function of these two proteins is to detect darkness and prevent inappropriate filamentation within the host, which would induce a potent immune response and pathogen clearance.

Host immunity and pathogen subversion

One of the most remarkable discoveries of recent years has been the extent to which cryptococci are able to manipulate the host immune response to dampen inflammation, avoid killing by phagocytic cells and ultimately disseminate into the CNS.

Inflammatory perturbation. In general, environmental fungi trigger a potent inflammatory response upon entry into the human host. By contrast, cryptococci appear to be immunologically inert, driving much lower levels of inflammatory cytokine release *in vitro* than other human fungal pathogens such as *C. albicans*[33]. This immunological masking relies on a variety of pathogen traits (**Figure 1**).

Firstly, the complex carbohydrates glucuronoxylomannan (GXM) and galactoxylomannan (GalXM), which make up most of the cryptococcal capsule, are extensively shed during infection and directly dampen inflammation by suppressing the pro-inflammatory NF-κB pathway and driving down levels of pro-inflammatory cytokines such as TNF[34]. In addition, emerging data indicate that cryptococcal chitin, and derivatives thereof, can also act to alter host inflammatory responses during infection[17]. Secondly, *Cryptococcus* blocks dendritic cell maturation by reducing both MHC class II-dependent antigen presentation and inhibiting the production of the pro-inflammatory cytokines interleukin (IL)-12 and IL-23 [35]. Lastly, via a series of as-yet poorly characterized steps, cryptococci are able to partially "repolarize" the immune response, at least in mice, from a strong Th1 response towards a weaker Th1 or often a Th2 response that is less effective at fungal clearance[17,36-38].

Collectively, these mechanisms generate an environment that is dominated by anti-inflammatory markers such as IL-4 and IL-33 [39,40,41] which, as a consequence, reduce cryptococcal killing by the immune system[38,42]. Therefore, modulating natural immune responses to cryptococcal infection towards a more pro-inflammatory profile offers one potential avenue for treatment. However, such approaches need to be carefully managed in order to avoid the potentially fatal "immune overreactions" that can accompany overt inflammation, which can be just as life-threatening as the original infection (**Box 4**).

Avoidance and escape from phagocytes. Following entry into the lung, the first immune cell typically encountered by cryptococci is a phagocyte such as an alveolar macrophage or dendritic cell. However, cryptococci are predisposed to avoid killing by these cells, due to their long evolutionary history of exposure to environmental amoebae (**Box 3**). Several cryptococcal virulence factors such as capsule synthesis, melanization and urease secretion combine to protect the

fungus from the harsh environment within phagocytic cells by neutralizing reactive oxygen species and pH, allowing it to survive and proliferate within such cells (**Figure 2**)[43].

More recently, it has also become clear that cryptococci exhibit a remarkable strategy to escape from within phagocytes. This process, which has been labeled vomocytosis or extrusion, involves inducing the fusion of the phagosomal membrane with the plasma membrane, which results in the expulsion of the fungi from the phagocyte[44-48]. In addition, either this process, or a closely related one, can drive the direct "lateral transfer" of cryptococci between host cells [44,45]. However, the underlying mechanisms of both of these remarkable processes remain unknown.

Although cryptococci employ several mechanisms to resist phagocytosis (such as through production of titan cells[15,49] and the assembly of a thick polysaccharide capsule), fungal uptake by phagocytes can still occur. However, if uptake does occur, cryptococci perturb both phagosome maturation[50] and modify the phagosome membrane in order to allow nutrient exchange and ultimately escape from within the host cell[51,52]. Notably, these effects are dependent on fungal virulence factors such as laccase and phospholipase B1. These enzymes have been classically thought of as having direct structural roles in melanin synthesis and membrane lipid modification, respectively, but the observation that they also mediate escape from phagocytosis suggests that laccase and phospholipase B1 may also have more subtle roles in modifying host signaling events[36,53,54].

Dissemination and entry into the CNS. A key feature of cryptococcal pathogenesis involves the exit of *Cryptococcus* from the lungs into peripheral blood circulation and entry into the CNS compartment. The CNS is both an immune privileged site and a highly sterile environment and thus *Cryptococcus* must have evolved potent methods to traverse the blood-brain barrier (BBB) and subsist in the CNS.

There are three proposed mechanisms that *Cryptococcus* could utilize to penetrate this impervious barrier. First, the yeasts could force their way between the tight junctions of the endothelial cells in a process known as

paracytosis, by using proteases such as Mpr1 to promote transmigration[55] (**Figure 2**). Impressively, when the *MPR1* gene was introduced into *Saccharomyces cerevisiae*, a fungus not normally able to penetrate the BBB, *S. cerevisiae* gained the ability to cross endothelial cells in an *in vitro* transwell assay, although the target of Mpr1 remains unknown. Additional studies utilizing powerful intravital imaging techniques demonstrated that cryptococci cross the BBB by inducing an embolic event in the microvasculature that lines the brain[56]. In essence, the initial "capture" of yeast within the brain is therefore passive, with the relatively large yeast cells becoming trapped at points where the blood vessel narrows. However, following the initial passive arrest, cryptococcal migration into the brain tissue is an active process, since it occurs only with live fungal cells and is dependent on the secretion of the cryptococcal enzyme urease[57]. To date, the part played by urease in this process remains enigmatic, although since urease produces ammonia, which is toxic towards mammalian cells, it is possible that urease acts to locally weaken the endothelial vessel wall, facilitating fungal entry.

The second mechanism of BBB penetration is transcytosis[58] (**Figure 2**). Hyaluronic acid situated on the surface of the cryptococcal cell binds to CD44 on the luminal endothelium, attaching the fungus to the host cell [59]. This binding then induces protein kinase C-dependent actin remodeling in the host cell, leading it to engulf the attached *Cryptococcus*[60]. Interestingly, recent work has revealed that the high levels of inositol present in the brain act as a trigger for this process, increasing hyaluronic acid expression in the fungus [61].

Finally, *Cryptococcus* is postulated to cross the BBB by a third method involving "hitchhiking" within host phagocytes, in a process termed the "Trojan Horse" hypothesis (**Figure 2**). This hypothesis is supported by the observation that depletion of alveolar macrophages in mice significantly reduces cryptococcal dissemination to the CNS[62], while infecting monocytes *in vitro* and transferring the cells into naïve hosts substantially increases cryptococcal accumulation in the brain compared to transferring *Cryptococcus* directly; both studies support the notion that phagocytes act as fungal carriers that breach the BBB[63]. Although paracytosis, transcytosis, and Trojan Horse models are all fundamentally different, it is reasonable to conclude that elements of each of

these models are readily observed and likely occur in concert during natural infection.

Not much is known about the physiology of *Cryptococcus* after it has traversed the BBB. However, a recent study of the transcriptome of cryptococcal yeasts isolated from cerebrospinal fluid (CSF) samples of patients offers some clues[64]. Most notably, *Cryptococcus* is remarkably metabolically active in the CSF *in vivo*, showing strong up-regulation of stress response genes and genes encoding enzymes that are involved in core metabolic processes; this is somewhat surprising, given that the CSF is a relatively nutrient depleted medium. By contrast, *Cryptococcus* growing in *ex vivo* CSF does not seem to be metabolically active, suggesting that the permanent cycling of CSF *in vivo* leads to a significantly higher nutrient content in the CSF than suggested by the analysis of *ex vivo* samples.

The modified fungal metabolism observed within the CSF is likely to have significant implications for pathogenesis. For instance, capsule synthesis is energetically highly demanding and there is a positive correlation between capsular size and severity of clinical disease [65]. Therefore, these data suggest that yeasts in a more active metabolic state may drive more aggressive CNS infections. Furthermore, fungal cells in different metabolic states are likely to give rise to different immune responses, which may also impact disease severity. In agreement with this possibility, the presence of a CSF inflammatory response consisting of an interplay of robust Th1 (IFN-γ and IL-6), Th2 (IL-4 and IL-10) and Th17 (IL-17) cytokines has recently been shown to be highly predictive of more rapid clearance of infection and consequently improved survival in patients with HIV-associated cryptococcal meningitis[66] (**Box 4**).

***Division of labour*.** The extent to which cryptococci can exploit phagocytic cells as a host has been strongly highlighted by the unusual cluster of cryptococcal disease now known as the Pacific Northwest Outbreak[67]. Although cryptococcosis is typically a disease of immunocompromised hosts, almost all of the human and animal cases within the Pacific Northwest Outbreak were immunocompetent hosts who became infected with near clonal strains of *C. gattii* from the VGII lineage. Both the epidemiology and etiology of these

infections differ from "classical" cryptococcosis (typically caused by C. *neoformans* in HIV-positive individuals)[33,68], which has led to vigorous efforts in order to establish the underlying mechanism driving virulence in the *C. gattii* VGII lineage.

The ability of the *C. gattii* VGII lineage to establish disease in individuals with a fully functional immune system seems to stem from a capacity to replicate extremely rapidly within host phagocytes (**Figure 2**), presumably overwhelming the host before adaptive immunity can be triggered[69]. Recent data has revealed that this rapid proliferation is, in turn, driven by a remarkable "division of labour" mechanism. In response to reactive oxygen species generated by the phagocyte, intracellular cryptococcal cells adopt different fates; some cryptococcal cells cease growth and acquire an unusual morphology characterized by extensive tubularisation of their mitochondria, whereas neighboring cells do not undergo this morphological transition. Notably, via a mechanism that remains unclear, the cells that undergo the morphological switch then protect neighboring cryptococci from the antimicrobial activity of the host phagocyte, enabling these cells to replicate rapidly, maximizing the proliferative capacity of the population as a whole[70]. These data highlight the *Cryptococcus*—phagocyte interaction as a key aspect of infection that may offer powerful opportunities for therapeutic intervention in both C. *neoformans* and C. *gattii* infections.

Anti-cryptococcal therapeutics

Despite its global distribution, treatment of cryptococcosis remains a major challenge, relying on a limited arsenal of decades-old therapeutic agents. Furthermore, therapeutic outcomes are generally poor and even with amphotericin-based therapy (to target *Cryptococcus*) and widespread access to anti-retroviral therapy (to target HIV, since most patients are immunocompromised HIV-positive patients), acute (3-month) mortality following cryptococcal meningoencephalitis remains 35-40%, both in resource-rich and resource-poor settings[71,72].

Currently used drugs. Only three classes of antifungal agents are currently used to treat cryptococcosis: polyenes (amphotericin B), azoles (fluconazole) and the pyrimidine analogue flucytosine (5FC) (**Figure 3**).

The cornerstone of treatment of cryptococcal meningoencephalitis is amphotericin B deoxycholate (AmBd), developed in the 1950s, which exerts its fungicidal effect both by binding to ergosterol in the cryptococcal cell wall (generating pores in the cell membrane) and by inducing cell death via oxidative damage [73-75]. AmBd is sometimes combined with 5-FC. The mechanism of action of 5-FC is deamination by the fungal enzyme cytosine deaminase into 5-fluorouracil (5-FU), which then acts via two pathways: 5-FU can be converted by cellular pyrimidine processing enzymes into 5-fluorodeoxyuridine monophosphate, which inhibits thymidylate synthetase and blocks DNA synthesis; or 5-FU can be converted into 5-fluorouridine triphosphate, which is incorporated into RNA, thereby disrupting protein synthesis and leading to growth arrest. AmBd and 5-FC act synergistically to produce the fastest rates of fungal clearance from CSF[76] and combination therapy results in a significant improvement in 10-week survival compared to treatment with AmBd alone[77]. This combination remains the recommended 'gold standard' induction treatment in international treatment guidelines[78] but presents significant challenges in resource poor settings, since AmBd must be administered intravenously and has notable toxicities. In addition, neither AmBd nor 5-FC are widely available in countries where cryptococcosis is most prevalent[79].

To circumvent the problems associated with AmBd and 5-FC combination therapies, the combination of fluconazole with 5-FC (which can both be administered orally) and shorter (1-week) AmBd-based induction treatment is being compared to the standard 2-week induction regimens in a multi-site phase III African trial [80]. Fluconazole is being tested because it has good oral bioavailability and excellent CSF penetration; these properties also make it recommendable for maintenance therapy after initial treatment. Fluconazole inhibits the fungal cytochrome P450 enzyme, 14α-demethylase, which is required for conversion of lanosterol to ergosterol, an essential component of the fungal cell membrane. However, fluconazole is as a fungistatic (rather than

fungicidal) making it is less effective at pathogen clearance and not recommended for initial therapy.

***Drug resistance*.** Resistance to antimicrobials is a growing issue in infectious disease and cryptococcosis is no exception. While environmental resistance is rare, acquired resistance has been observed with all three classes of antifungals in use against *Cryptococcus* species.

Polyene resistance is uncommon but has been reported in *C. neoformans*, with mutations in sterol synthesis and therefore alteration of the target site noted in isolates with extensive exposure to AmB [81]. For 5-FC, single mutations at varying points along the 5-FU intracellular pathways lead to *in vitro* and clinical resistance. Therefore, monotherapy with 5-FC is not appropriate due to rapid selection of resistant *Cryptococcus* leading to treatment failure; the drug is thus always combined with either AmB or fluconazole. Fluconazole, like 5-FC, is fungistatic, making it liable to evolution of secondary resistance during prolonged treatment[82]. A key mechanism of resistance against fluconazole is the selection of intrinsically resistant cryptococcal sub-populations [83] that carry specific chromosomal disomies [84] and thus overexpress the *ERG11* gene (which encodes the fluconazole target enzyme lanosterol-14α-demethylase[85]) or have enhanced drug efflux by the ATP Binding Cassette (ABC) transporter-encoding gene *C. neoformans* AntiFungal Resistance 1 (*CnAFR1*) [86].

New drugs. Given the ongoing high global incidence and mortality from cryptococcal meningoencephalitis, the dearth of drugs, together with toxicity and the potential for development of resistance, there is an urgent need for new drugs. Recent activity in this area has begun to highlight potential routes either for the discovery of novel antifungals or for the repurposing of existing molecules showing anti-cryptococcal activity (**Figure 3**).

An ideal antifungal drug should be fungal-specific, to avoid host cell toxicity; this is challenging, given that fungal cellular processes are more closely related to mammals than those that are targeted by common antimicrobials, such as the ones used to target bacterial pathogens. Furthermore, an ideal antifungal drug should target either a virulence factor or a fungal component

essential for fungal viability. Such a drug should be fungicidal when used alone or when combined with the widely available fluconazole, should have good oral bioavailability (allowing it to be readily administered even in resource-poor settings) and be able to enter cryptococcal niches within the host (such as phagocytes and the CNS).

One obvious target of such a drug is the cryptococcal cell wall. Unfortunately, the latest class of antifungals active against the cell wall, the β-1,3-D-glucan synthase inhibitors (echinocandins), have no significant anti-cryptococcal activity. However, synthesis of another cell wall component, glycosylphosphatidylinositol (GPI)-anchored mannoproteins, is inhibited by the orally-active experimental molecule E1210, which has *in vitro* activity against *Cryptococcus* and other medically-relevant fungi (such as *Candida* and *Scedosporium* species) and is currently in pre-clinical development[87].

Further along the development pipeline is VT-1129, an orally-available ergosterol synthesis inhibitor which shows good CNS penetration and is fungicidal in murine models of *Cryptococcus* infection. VT-1129 blocks the activity of CYP51, an essential enzyme in the pathway to produce ergosterol, and is currently entering human clinical trials [88]. Also in Phase I trials is the arlyamidine T-2307, which targets the fungal mitochondrial membrane[89]. T-2307 is a fungicidal injectable compound that shows comparable efficacy to AmB in murine models of infection.

Given the lack of market forces driving pharmaceutical development for a neglected disease such as cryptococcal meningoencephalitis, an alternative, cheaper and more expedient strategy in drug development is the repurposing of drugs not originally developed for antifungal use. Recently developed high-throughput screening techniques have advanced the repurposing effort. One such powerful tool is chemical-genetic profiling, whereby large collections of cryptococcal knockout mutants, for which the function of a particular pathway is compromised, are screened against a library of small molecules[90], and the growth behavior of the screened strain (i.e. increased or decreased susceptibility) is then recorded. This technique was recently performed with 1448 knockout mutants of *C. neoformans* and demonstrated distinct differences in drug susceptibility between this species and the model organism

Saccharomyces cerevisiae which, until now, has been the standard choice for such screens[90]. As proof of principle, this approach has identified a number of molecules that synergize strongly with fluconazole to inhibit ergosterol synthesis in *C. neoformans* and which are now being further investigated for potential clinical applicability. Moreover, this method has the additional advantage of providing information on the mechanism of action of lead compounds and can therefore identify both potential new drugs and potential new drug targets.

A more classical approach is to screen for compounds that trigger fungal lysis (detected by the release of adenylate kinase, a cytosolic enzyme, into the medium) or alter ATP content [91] (a particularly effective approach for identifying compounds that are antifungal under starvation conditions). This strategy has identified a collection of off-patent drugs[92] with anti-cryptococcal activity that are additive or synergistic with fluconazole. These include drugs as diverse as amiodarone (a cardiac anti-arrhythmic drug), phenothiazines (widely used antipsychotics) and tamoxifen (an estrogen antagonist used in the treatment of breast cancer). Illustrating the utility of these approaches, tamoxifen in combination with fluconazole, decreased the *C. neoformans* burden in the brain by ~1 $\log_{10}$ CFU per gram of brain tissue, in a mouse model of infection[93]. Finally, another candidate that has emerge from repurposing screens is the anti-depressant sertraline (also known as Zoloft®), a drug that is fungicidal, has high CNS penetration, and appears to target fungal protein synthesis through an unknown mechanism[94]. Sertraline is currently being evaluated in combination with AmBd and fluconazole in a phase II/III clinical trial [95].

Outlook

The last five years have seen a remarkable revolution in our understanding of cryptococcosis. A deeper understanding of the natural ecology and an appreciation of the genetic and phenotypic diversity of this group of pathogens is transforming our understanding of cryptococcal pathogenesis. Meanwhile, huge progress has been made in understanding the host immune response to infection and how this process is hijacked by cryptococci to drive latency, dissemination and proliferation. However, despite these advances, cryptococcosis remains a

major worldwide killer, causing hundreds of thousands of deaths per year and the anti-cryptococcal drug arsenal remains limited. To address this, there is renewed focus on translational research to discover and develop new therapeutic agents and to evaluate new therapeutic strategies in a clinical setting. Whilst progress is being made in this respect, more is urgently required, and advances in understanding of the pathogenesis of *Cryptococcus spp* offer new opportunities for developing therapeutics beyond the traditional approaches of killing the fungal cell or preventing its replication. In particular, the rapidly expanding understanding of the *Cryptococcus*-host interface opens up new avenues for potential therapy development; for instance, in modifying host inflammatory responses, augmenting phagocytic clearance of the fungus, disrupting population signaling or preventing migration to the CNS. Together, such approaches offer the hope of significantly reducing the huge global burden of infection and making fatal cryptococcosis a disease of the past.

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Box 1. Clinical cryptococcosis.

Epidemiology. Since cryptococci are capable of extended latency within host cells[43] and most humans encounter the organism in early childhood[8], it has been assumed that most clinical cases represent reactivation of a longstanding, asymptomatic infection (triggered, for instance, by falling $CD4^+$ T-cell counts in HIV-infected individuals). The proportion of clinical disease representing reactivated latent disease versus primary infection is unknown in HIV-positive individuals, but a study in patients with cryptococcosis following solid-organ transplantation found only 52% of infections to be due to reactivation[96], suggesting that the classical view of cryptococcosis as a reactivating infection may not be accurate.

Emerging data are also highlighting the heterogeneity of cryptococcal disease worldwide, as illustrated by the prevalence of serum cryptococcal antigen (CrAg) in HIV-positive cohorts in different countries (see the figure, which displays the highest recorded prevalence per country). In addition, it is now clear that there is also considerable global heterogeneity in the fungal population structure. For example, *C. neoformans* var *grubii* (serotype A) is the predominant global cause of HIV-associated cryptococcal meningoencephalitis, but in China this organism frequently infects apparently immunocompetent hosts[97]. Similarly, particular lineages of *C. neoformans* vary both in virulence[98-100] and in their ability to infect immunocompromised or immunocompetent individuals[101]. In the near future, intensive whole genome sequencing efforts for both cryptococcal isolates and affected patients offers the possibility of being able to explain the relative contribution of host and pathogen genotypes underlying these global patterns of disease.

Susceptibility. In contrast to other systemic fungal infections (such as candidiasis), relatively little is known about genetic risk factors for cryptococcosis. However, recent allelic association studies have shown that apparently immunocompetent individuals with cryptococcosis are significantly more likely to have defects in mannose-binding lectin[102] or be homozygous for the "232I" allele of the Fcgamma receptor 2B (FcgR2B)[103], although these polymorphisms are relatively common and thus, on their own, are clearly not sufficient to render an individual fully susceptible to cryptococcosis. Therefore,

subtle defects in the innate immune response to fungi may underlie at least some cases of C. *neoformans* infection in otherwise healthy individuals. Similarly, in HIV-positive patients, allelic variation in a different FcgR, FcgR3A, also correlates with susceptibility[104]. In this case, individuals with a higher affinity receptor variant are at greater risk of infection, perhaps indicating that efficient uptake of the pathogen may actually aid dissemination and drive more severe disease. This is particularly striking since the same is true from the pathogen perspective; cryptococcal strains that are more avidly phagocytosed drive more aggressive disease and carry a higher risk of death in patients[105]. Thus, excessive phagocytosis as a result of either host or pathogen variation appears to drive cryptococcal dissemination, strongly supporting the "Trojan Horse" model of pathogen spread (see the main text).

Diagnosis. Diagnosis of cryptococcosis relies on detection either of the organism itself or its shed capsular GXM polysaccharide in serum or CSF. This has been hugely facilitated by the introduction of the point-of-care lateral flow cryptococcal antigen assay, which is cheaper and more sensitive than earlier serological tests[106]. This test can detect very early dissemination and has facilitated cohort studies across the world, revealing a prevalence of cryptococcal antigens in HIV-infected patients ranging between 2 and 21%. As an increasing proportion of cases of cryptococcal meningoencephalitis are now presenting as "unmasking" of latent infection following therapy (i.e. the appearance of clinical symptoms following immune reconstitution by antiretroviral treatment), wider implementation of a 'screen-and-treat' approach is cost effective as a public health intervention and has been demonstrated to reduce mortality in African HIV cohorts in the first year on ART[107].

Box 2: The evolutionary history of cryptococci

The two *Cryptococcus* species, *C. gattii* and *C. neoformans*, probably diverged from a common environmental saprophyte ancestor around 30-40 million years ago[108,109] (see the figure). For *C. neoformans,* extensive genetic data now indicates a common origin in sub-Saharan Africa[5,110,111]. The observation that most non-African *C. neoformans* populations are near-clonal supports a model in which recombining African populations of cryptococci occasionally dispersed to

other parts of the globe. Coalescence analyses indicate that almost all of these events have occurred within the last 5000 years, suggesting the potential involvement of human or avian migrations in this process[5].

Probing the origin and diversity of C. *gattii* has proven more challenging. There is a growing consensus that the evolutionary origins of this species lie within Australia and South America, since most dispersed lineages of C. *gattii* are near clonal (such as the lineage responsible for the Pacific Northwest Outbreak) but always cluster with Australian and South American isolates during phylogenetic analyses, with an estimated origin within the last 50 thousand years [112-114]. A recurrent theme therefore appears to be that local populations of *C. gattii* in endemic areas (such as Brazil) undergo continual recombination, which occasionally results in a novel recombinant lineage that disperses and expands rapidly by means of clonal growth (either asexual cell division or same-sex mating) [112,113,115].

Both species of *Cryptococcus* have a bipolar mating system in which cells are either mating type a (MATa) or mating type alpha (MATα) (reviewed in [116]). Classical mating involves genetic exchange between a MATa and MATα strain, followed by normal Mendelian segregation of alleles. However, both species of cryptococci are also capable of same-sex mating in which two strains of the same mating type are able to exchange genetic material [115,117]. In addition, diploid and aneuploid strains are not uncommon[118,119], and inter- and intra-species hybrids can be found both in the environment and in patients[120,121]. Thus the global population structure of these pathogens reflects a complex mix of "diversity generating" recombination and aneuploidy, coupled with highly clonal amplification steps during dispersion events.

Box 3: The evolution of virulence in cryptococci

Opportunistic pathogens represent an evolutionary enigma: why has natural selection driven the acquisition of often highly specific virulence factors when the majority of the population remain as exclusively environmental organisms for their entire existence? This conundrum is particularly pertinent for cryptococci, which are abundant in the environment and yet are remarkably well suited to survive in a human host.

A compelling hypothesis to resolve this conundrum is that of "accidental pathogenesis"[122]. This hypothesis proposes that cryptococcal pathogenesis does not result from direct selection for virulence within a mammalian host, but rather by the evolution of traits (which happen to be advantageous in mammals) in response to other selective pressures in both environmental and animal niches. So, for instance, the complex polysaccharide capsule, laccase activity and ability to synthesize melanin, which are all *Cryptococcus* virulence factors, are likely to offer protection against environmental pressures such as desiccation or exposure to ultraviolet light [123], or aid in the colonization of plant hosts [124]. Similarly, cryptococci can replicate not only within vertebrate phagocytes, but also within free-living phagocytic amoebae[125] (see the figure). Despite the enormous evolutionary distance between vertebrates and amoebae, many of the mechanisms used by phagocytic white blood cells to kill pathogens (e.g. the generation of reactive oxygen species or secretion of antimicrobial peptides) are identical to those used by amoebae to digest ingested prey. Thus, over millions of years, cryptococci have been selected to evolve strategies that facilitate fungal growth and persistence within amoebae that coincidentally also enable their survival within phagocytes. Such strategies include not only stress-tolerance approaches, such as resistance to reactive oxygen species[126], but also elaborate mechanisms to regulate expulsion from host cells[46,47].

In addition, *Cryptococcus* has a remarkable ability to perturb adaptive immunity, preventing complete fungal clearance and resulting in latent infections.[19,127]. Perhaps the ability to remain latent without perturbing its host is the strongest evidence for host adaptation by *Cryptococcus*. Since only higher vertebrates have adaptive immune systems, *Cryptococcus* species probably evolved these properties under the selective pressures of reptilian, avian or mammalian hosts within the environment, which also explains the diverse range of animals that can succumb to cryptococcosis.

Taken together, these observations suggest that interactions with both soil microorganisms (such as amoebae and nematodes) and vertebrates likely have a critical role in the virulence potential of *Cryptococcus* (reviewed in [128]). Intriguingly, laboratory studies have shown that selection pressure by amoebae can rapidly select for resistant, pseudohyphal forms of cryptococci[23]. These

forms are attenuated in mammalian hosts and consequently frequently revert to yeast upon entry into a vertebrate host. Thus, rapid microevolutionary events may have an important role in driving cryptococcal pathogenesis in different hosts.

The paradigm of 'accidental pathogenesis' extends beyond cryptococci to other fungal[129] and even bacterial pathogens[130], such as *Aspergillus, Blastomyces* and *Legionella* species, and highlights two important issues. Firstly, as pathogens adapt to changing environments due to global warming, we may see additional instances of "accidental pathogenesis" through the selection of new traits that promote both environmental survival and pathogenesis in humans. Secondly, we should be alert to the fact that changes in human behavior and habitat use (e.g. increased tourist access to remote rainforest or desert areas) may expose us to novel potential pathogens that have been predisposed to infection via selection through environmental predators.

Box 4. Host immunity: too little or too much?

Poor inflammatory responses to cryptococci, such as those in patients with advanced HIV infection, lead to life-threatening meningoencephalitis. Consequently, immune profiling of patient peripheral T-cell responses and CSF cytokines has shown that those mounting a pro-inflammatory immune response are more likely to clear the pathogen and survive infection[66]. Moreover, augmenting pro-inflammatory immune responses using adjunctive IFN-γ improves fungal clearance[131]. Conversely, individuals producing anti-cytokine antibodies that interfere with appropriate inflammatory responses are known to be at enhanced risk of infection[132].

Although a potent immune response to *Cryptococcus* is clearly essential for fungal clearance, too strong a response can also be harmful. For instance, a low level of anti-inflammatory activity driven by both Th2 and regulatory T cells[133,134] prevents complete immune paralysis. This is also the case for the classical antifungal cytokine IL-17, which is essential for resistance to cryptococcosis[135] but whose effects must also be regulated by IL-23 in order to prevent damage to the host due to excessive inflammation[136]. Thus a "successful" immune response to cryptococcal infection appears to be a complex blend of

Th1, Th2 and Th17 responses, which must be counter-regulated to prevent either runaway fungal growth or damaging levels of inflammation.

This critical role for "restraining" inflammatory signaling is particularly highlighted by the problem of immune reconstitution inflammatory syndrome (IRIS). This life-threatening inflammatory reaction occurs in some HIV-infected patients during antiretroviral therapy (ART) and is attributable to the newly reconstituted immune system "overreacting" to residual pathogen antigen. Consequently, the timing of clinical intervention is critical; early introduction of ART is important to restore cell-mediated immunity, but if introduced too early (during the initial 2 weeks following induction of antifungal treatment) at a time of high fungal load, the risk of death is increased[137]. Development of IRIS is particularly likely in patients whose initial pro-inflammatory response to cryptococcal infection is poor, resulting in high residual antigen burden[138]. Coupled with an exaggerated baseline CNS chemokine response, this results in aberrant CNS immune responses following ART initiation, resulting in IRIS.

Excessive inflammation can also occur following withdrawal of immune suppression in solid organ transplant recipients, as well as in apparently immunocompetent patients. In such situations, steroids are often administered alongside antifungals. It remains unclear, however, whether steroids are beneficial in other contexts: a multi-centre clinical trial to address this issue (investigating the effect of adjunctive dexamethasone in patients with HIV-associated cryptococcal meningoencephalitis) has been terminated early and results are awaited[139].

691

692 **Figure 1. Inflammatory signaling in response to cryptococcal infection.**
693 Cryptococci inevitably shed microbial molecules that contain pathogen
694 associated molecular patterns (PAMPS). Such fungal molecules are typically cell
695 wall or capsular components such as chitin, β-glucan or glucuronoxylomannan
696 (GXM), which are detected by immune sentinel cells, most notably dendritic cells
697 (DCs). DC activation then summons T-cell help, inducing $CD4^+$ T-cells to secrete
698 cytokines that induce a T helper cell 1 (Th1) response (such as interleukin (IL)-
699 12 and IL-23). Th1 cells produce pro-inflammatory cytokines (such as IFN-γ)
700 that ultimately control fungal infection. However, some fungal PAMPs can
701 influence DC activation, including modulating the levels of MHC-II or NF-kB
702 signaling. This leads to the generation of a Th2 response (mediated by the
703 production or cytokines such as IL-4 and IL-33); this anti-inflammatory
704 environment impacts the ability of macrophages to mediate fungal clearance.
705

706 **Figure 2. Infection establishment and dissemination within the human**
707 **host.** Cryptococcal cells typically enter the human host through the lung. Here
708 they are recognized by patrolling phagocytes but can avoid uptake either by
709 growing into very large "Titan" cells, or by relying on the antiphagocytic
710 properties of the fungal capsule. If uptake occurs, however, cryptococci are able
711 to survive and persist within phagocytes. For most strains, a failure in host
712 immune function is then required to allow intracellular proliferation. However,
713 the unusual Pacific Northwest Outbreak (PNO) strains of *C. gattii* can proliferate
714 within immunocompetent host cells by exploiting a poorly-characterized
715 "Division of Labour" mechanism: in response to reactive oxygen species
716 generated by the phagocyte, some cryptococcal cells acquire an unusual
717 morphology characterized by extensive tubularisation of their mitochondria,
718 which increases survival of neighboring cells (via a mechanism that remains
719 unclear). *Cryptococcus* proliferation within phagocytes ultimately leads either to
720 host cell lysis or to a novel non-lytic escape mechanism termed vomocytosis.
721 Upon replication in the lung, cryptococci are able to disseminate to other tissues,
722 including the central nervous system (CNS). Entry into the CNS can occur in
723 three ways: by squeezing between host endothelial cells (paracytosis), which

724 involves the fungal protease Mpr1; by moving directly through endothelial cells
725 (transcytosis), in a process that is mediated by hyaluronic acid in the fungal
726 capsule and the host receptor CD44; or by "hitching a ride" within migrating
727 phagocytes, in a process termed the "Trojan horse" hypothesis.
728
729 **Figure 3: Current and future therapies for cryptococcosis.** Schematic
730 representation of a cryptococcal cell, showing key current and potential
731 therapeutic targets and examples of antifungal drugs acting at each site. Drugs in
732 current clinical use are shown in red, novel drugs are shown in blue and
733 repurposed drugs are shown in green. The three classes of antifungal agents
734 currently used to treat cryptococcosis are polyenes (amphotericin B), azoles
735 (fluconazole) and the pyrimidine analogue flucytosine (5-FC) Amphotericin B
736 deoxycholate (AmBd) acts by binding to ergosterol in the cryptococcal cell wall,
737 generating pores in the cell membrane, and by inducing cell death via oxidative
738 damage. 5-FC is deaminated by the fungal enzyme cytosine deaminase into 5-
739 fluorouracil (5-FU), which then inhibits thymidylate synthetase and blocks DNA
740 synthesis or is converted into 5-fluorouridine triphosphate, which is
741 incorporated into RNA and disrupts protein synthesis. Fluconazole inhibits the
742 fungal cytochrome P450 enzyme, 14α-demethylase, which is required for
743 conversion of lanosterol to ergosterol, an essential component of the fungal cell
744 membrane. E1210 inhibits the synthesis of the cell wall component
745 glycosylphosphatidylinositol (GPI)-anchored mannoproteins. VT-1129 blocks
746 the activity of CYP51, an essential enzyme in the pathway to produce ergosterol.
747 The arlyamidine T-2307 targets the fungal mitochondrial membrane. Tamoxifen
748 (an estrogen antagonist used in the treatment of breast cancer) targets
749 calmodulin and the anti-depressant sertraline appears to target fungal protein
750 synthesis through an unknown mechanism.
751
752

GLOSSARY

Pacific Northwest Outbreak – an unusual cluster of cryptococcal disease in otherwise healthy (rather than immunocompromised) individuals. First identified on Vancouver Island, British Columbia, in 1999 (and hence originally called the Vancouver Island Outbreak), both the causative organism and cases of human and animal disease have now expanded into mainland Canada and the northwestern USA, prompting a renaming of the outbreak.

Iatrogenic – caused by medical treatment. For instance, infections due to contaminated surgical instruments.

Zoonotic – a disease transmitted from non-human animals to people

Diploid – having two homologous sets of chromosomes, one from each parent

Aneuploid – having an 'unbalanced' set of chromosomes; for instance, having only a single copy of one chromosome in an otherwise diploid genome.

Polyploid – having multiple (more than two) sets of homologous chromosomes

Founder – the initial (small) group of individuals that seeds a new population. For instance, the inoculum that starts an infection, or the first individuals to arrive on a new island habitat.

Quorum sensing – the regulation of gene expression or behavior in response to changes in the local population size.

Paracrine – a signal that acts close to where it is produced; for instance, on neighbouring cells.

Filamentation – the growth of an organism by elongation without division.

MHC Class II – molecules that are expressed on the surface of professional antigen presenting cells (such as macrophages and dendritic cells) and present extracellular antigens to the immune system to coordinate an immune response

Th1/Th2 response – A broad characterization of the differentiation of $CD4^+$ helper T cells (Th). Th1 responses are generally provoked by intracellular pathogens and Th2 responses are typically involved in the elimination of parasitic worms, harmful allergic responses, and dampening of Th1-mediated inflammation. In the context of cryptococcal infection, Th1 responses are widely thought to be protective and Th2 responses are detrimental.

Melanization – the production of the dark, insoluble pigment melanin, which provides protection from high energy radiation and reactive oxygen molecules.

Blood-brain barrier – a specialized endothelial barrier that prevents the entry of cells or large molecules into the central nervous system

Paracytosis – transitioning between tissues by moving between, rather than through, adjacent cells.

Transcytosis - transitioning between tissues by moving directly through cells, rather than between adjacent cells.

Hyaluronic acid – an abundant, high molecular weight polysaccharide that forms part of the extracellular matrix, particularly in neural tissue.

Cerobrospinal fluid (CSF) – a clear fluid produced in the brain which bathes the central nervous tissue and is slowly turned over.

Fungistatic – an antimicrobial agent that prevents fungal growth, but does not kill the organism

Fungicidal – an antimicrobial agent that kills fungi, rather than simply preventing growth

Coalescence analysis – an evolutionary analysis method in which genetic drift is "played backwards" in order to calculate common ancestry of individuals within a population and thereby estimate lineage branch points within an evolutionary phylogenetic tree.

Bipolar mating – a system to control sexual reproduction that relies on a single genetic locus at which individual organisms can carry one of two alleles, effectively generating a species with two sexes.

Regulatory T-cell **–** a type of T-cell that functions to regulate the immune system, typically by suppressing the function of proinflammatory effector T-cells.

Online only

Author bios

Kirsten Nielsen received her PhD from North Carolina State University and then joined the medical mycology community while pursuing post-doctoral training at Duke University. Kirsten is currently an Associate Professor in the Department of Microbiology and Immunology at the University of Minnesota, where her research program focuses on factors influencing fungal pathogenesis both in animal models and during human diseases.

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cryptococcosis, host genetic susceptibility to cryptococcal infection and the evolution and mechanisms of fluconazole resistance in *Cryptococcus* sp.

Neil Stone is a Specialist Registrar physician in Infectious Diseases and Microbiology and is a Wellcome Trust Clinical Research Fellow at the Institute of Infection and Immunity at St. George's, University of London, with an interest in neglected infections in the developing world and cryptococcal meningitis in particular. He is currently undertaking a clinical PhD under the supervision of Dr Tihana Bicanic and Professor William Hope, investigating the evolution of fluconazole resistance in a cohort of patients with cryptococcal meningitis in Tanzania, East Africa.

Online Summary

- Cryptococcosis is a widespread opportunistic fungal infection of humans and other animals.
- *Cryptococcus* species that infect humans likely evolved as "accidental pathogens" in response to environmental selective pressure.
- Recent genomic analyses have highlighted the evolutionary history of *Cryptococcus* species and narrowed down the geographical origin of an unusual, hypervirulent outbreak.
- Despite being accidental pathogens, cryptococci display a remarkable ability to manipulate the human immune response in order to facilitate disease establishment and spread.
- Detailed *in vivo* and *in vitro* characterization of *Cryptococcus* species has started to elucidate the details of multiple mechanisms of pathogenesis that likely have important roles in disease severity. These include changes in fungal morphology, interactions with host phagocytes and mechanisms that allow *Cryptococcus* to disseminate from the lung to the CNS.
- Renewed efforts to develop improved therapeutic approaches have highlighted potential new drugs and potential new uses for old drugs in the fight against cryptococcal disease.

ToC blurb

Recent studies have elucidated multiple virulence mechanisms used by *Cryptococcus* to infect, disseminate within and ultimately kill their human host. In this Review, May *et al*. describe these recent developments in understanding host-fungal interactions, discuss how they affect disease severity and debate current and future therapeutic interventions against cryptococcosis.

Subject categories

Biological sciences / Microbiology / Fungi / Fungal pathogenesis

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